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# Identification of Microtubule-associated Proteins in The Centrosome, Spindle, and Kinetochore of The Early *Drosophila* Embryo

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**Abstract.** We have developed affinity chromatography methods for the isolation of microtubule-associated proteins (MAPs) from soluble cytoplasmic extracts and have used them to analyze the cytoskeleton of the early *Drosophila* embryo. More than 50 *Drosophila* embryo proteins bind to microtubule affinity columns. To begin to characterize these proteins, we have generated individual mouse polyclonal antibodies that specifically recognize 24 of them. As judged by immunofluorescence, some of the antigens localize to the

mitotic spindle in the early *Drosophila* embryo, while others are present in centrosomes, kinetochores, subsets of microtubules, or a combination of these structures. Since 20 of the 24 antibodies stain microtubule structures, it is likely that most of the proteins that bind to our columns are associated with microtubules in vivo. Very few MAPs seem to be identically localized in the cell, indicating that the microtubule cytoskeleton is remarkably complex.

MICROTUBULE-ASSOCIATED proteins (MAPs)<sup>1</sup> are thought to play a central role in determining the structure and function of microtubule networks in eukaryotic cells. MAPs were originally identified in mammalian brain tissue extracts as abundant proteins that cosediment with microtubules (for reviews, see Vallee et al., 1984; Olmstead, 1986). More recently, similar criteria have been used to identify MAPs in a number of nonneuronal systems (Vallee and Collins, 1986; Goldstein et al., 1986; Lye et al., 1987).

None of the MAPs that cosediment with microtubules have been found to localize to the centrosome or the kinetochore, two extensively studied microtubule-organizing centers in the cell. Most MAPs that have been identified thus far are relatively abundant proteins that bind along the entire length of microtubules (Vallee and Bloom, 1983; Bloom et al., 1984; Huber and Matus, 1984; Binder et al., 1985). MAPs that function as components of the centrosome or the kinetochore would be expected to be present only near the ends of microtubules, and are therefore likely to be much less abundant. These low abundance MAPs may be difficult to detect by current methods.

We have developed procedures for the isolation of MAPs by microtubule affinity chromatography, with the hope that even minor proteins that bind to microtubules can be identified and characterized. Previous work has shown that affinity chromatography is a powerful technique for the identification, purification, and characterization of interact-

ing proteins (for examples, see Formosa and Alberts, 1984; Dedhar et al., 1987). We have used our technique to isolate a large number of previously uncharacterized MAPs from the early *Drosophila* embryo, which seems to provide an especially good model system for studying cytoskeletal functions.

The *Drosophila* embryo begins development as a giant syncytial cell. The earliest nuclear divisions occur in the interior of the embryo, but, after nine nuclear divisions, the majority of the nuclei have migrated to the cortex where they form an evenly spaced monolayer. The nuclei in this monolayer divide four more times and then become synchronously cellularized by invaginations of the plasma membrane to form the cellular blastoderm (Rabinowitz, 1941; Zalokar and Erk, 1976; Foe and Alberts, 1983). The nuclear divisions that precede this cellular blastoderm stage take place at intervals of 8–20 min, and the dynamic rearrangements of the microtubule arrays during each nuclear cycle can be observed in living embryos after injection of fluorescently-labeled tubulin subunits (Kellogg et al., 1988). Large quantities of early embryos are readily available for biochemical analyses, and these embryos are also amenable to immunofluorescence, microinjection, and genetic studies. These factors combine to make the early *Drosophila* embryo an attractive system in which to study the morphogenesis of microtubule arrays and their role in embryonic development.

To begin a characterization of the affinity-purified *Drosophila* embryo MAPs, we have made a library of mouse polyclonal antibodies that specifically recognize 24 of them. These antibodies have allowed us to determine the subcellular localizations of the affinity-purified proteins in embryos.

1. Abbreviations used in this paper: MAP, microtubule-associated protein; TAME, *N*-*p*-tosyl arginine methyl ester.

The results suggest that the majority of the many proteins that bind to microtubule affinity columns are associated with microtubules in the cell, and they have allowed us to identify MAPs that localize to centrosomes and kinetochores.

## Materials and Methods

### Materials

All chemicals used were reagent grade. *N*-*p*-tosyl arginine methyl ester (TAME), pepstatin A, leupeptin, aprotinin, and Freund's adjuvant were from Sigma Chemical Co. (St. Louis, MO). Rhodamine-conjugated goat anti-mouse antibody was from Cappel Laboratories (Malvern, PA), and alkaline phosphatase-conjugated goat anti-mouse antibody was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Monoclonal antibodies against alpha and beta tubulin were from Amersham Corp. (Arlington Heights, IL). Taxol was a generous gift of Dr. Matthew Suffness (National Institutes of Health).

### Buffers and Stock Solutions

Protease inhibitor stock: 1 mM benzamidine-HCl, 0.1 mg/ml phenanthroline, 1 mg/ml each of aprotinin, leupeptin, and pepstatin A (this stock is used at dilutions of 1:100–1:1,000, as noted). PMI buffer: 0.1 M Pipes-KOH, pH 6.8, 2 mM Na<sub>3</sub> EGTA, 1 mM MgCl<sub>2</sub>, 1 mM GTP, 1 mM TAME. PMIX buffer: PMI buffer plus 0.5 mM DTT and protease inhibitor stock (1:100). PMIX.1 buffer: as above, but with 1:1,000 dilution of protease inhibitor stock. C Buffer (column buffer): 50 mM Hepes-KOH, pH 7.6, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub> EGTA. CX buffer: C buffer supplemented with 10% glycerol, 25 mM KCl, 0.5 mM DTT, and protease inhibitor stock (1:1,000). BRB80 buffer (microtubule assembly buffer): 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub> EGTA. BRB80X buffer: BRB80 supplemented with 0.5 mM DTT, 1 mM GTP, 5  $\mu$ M taxol, and protease inhibitor stock (1:100). PBS: 10 mM sodium phosphate, pH 7.3, 0.15 M NaCl. TBS: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl. Polyacrylamide gel sample buffer: 63 mM Tris-HCl, pH 6.8, 3% sodium dodecylsulfate (SDS), 5%  $\beta$ -mercaptoethanol, 10% glycerol.

### Purification of *Drosophila* Tubulin

Tubulin is purified from early *Drosophila* embryos by procedures modified from those used by Detrich and Wilson (1983) to purify tubulin from sea urchin embryos. As starting material, we use 0–3-h collections of embryos that have been frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Frozen chunks of embryos are placed between several layers of aluminum foil, crushed with a hammer, and added to 2 vol of PMI buffer at  $15^{\circ}\text{C}$  containing protease inhibitor stock (1:100). After stirring briefly, PMSF is added to 1 mM, and the embryos are homogenized by several passes of a motor-driven teflon dounce at  $4^{\circ}\text{C}$ . After the crude homogenate is clarified by centrifugation at 100,000 g for 70 min, the supernatant is brought to 0.5 mM DTT and mixed with 0.5 vol of packed phosphocellulose (Whatman Inc., Clifton, NJ), previously equilibrated with PMIX buffer. After 30 min of gentle mixing on a rotator, the phosphocellulose resin is pelleted by centrifugation for several min at 1,000 g, and the supernatant is saved. The phosphocellulose is washed twice with 0.5 vol of PMIX buffer, and these supernatants are combined with the first supernatant.

The tubulin in the above supernatants is bound to DEAE cellulose by adding the supernatant to 0.33 vol of packed DEAE cellulose (DE52; Whatman Inc.) previously equilibrated with PMIX buffer. After gently mixing the DEAE cellulose and the extract on a rotator for 30 min, the DEAE cellulose is pelleted and washed two times with 0.5 vol of PMIX buffer. A slurry of the resin is poured into a column (3.5-cm-diam for 150 ml of DEAE cellulose) and washed with 2 column vol of PMIX.1 buffer containing 0.15 M NaCl. Tubulin is then eluted from the DEAE cellulose column with PMIX.1 buffer containing 0.4 M NaCl. Fractions of 2–3 ml are collected at a flow rate of 2 column vol/h. The eluted fractions are assayed for protein (Bradford, 1976), and peak fractions are pooled and dialyzed for 40 min against 10 vol of BRB80 buffer containing 0.5 mM DTT, followed by another 40 min against fresh buffer. If the protein concentration is below 2 mg/ml at this point, the solution is concentrated using an ultrafiltration device (Amicon Corp., Danvers, MA). Tubulin is polymerized into microtubules by addition of GTP to 1.0 mM, MgCl<sub>2</sub> to 4 mM, and DMSO to 10%, followed by incubation at  $25^{\circ}\text{C}$  for 40 min. The microtubules are collected by cen-

trifugation through a 50% sucrose cushion in BRB80 at 150,000 g for 60 min at  $25^{\circ}\text{C}$ . The microtubules are resuspended to  $\sim 5$  mg/ml in BRB80 and depolymerized by incubation on ice for 30 min. After insoluble material is removed by centrifugation at 100,000 g for 30 min, the tubulin solution is either quickly frozen on liquid nitrogen and stored at  $-70^{\circ}\text{C}$  or used directly for column construction (see below). All steps are carried out at  $4^{\circ}\text{C}$ , except where noted.

### Taxol-induced Assembly of Microtubules

To form taxol-stabilized microtubules for the construction of microtubule affinity columns, tubulin at 2–3 mg/ml in BRB80 is assembled into microtubules by addition of 1 mM GTP, followed by step-wise addition of taxol. The taxol-induced polymerization of tubulin is carried out gradually in order to prevent the formation of aberrant structures (Schiff et al., 1979). An initial aliquot of taxol is added to bring the taxol concentration to 0.15  $\mu$ M, followed by a 10-min incubation at  $25^{\circ}\text{C}$  (*Drosophila* tubulin) or  $37^{\circ}\text{C}$  (bovine tubulin). Three additional aliquots of taxol are then added over a 25 min period to bring the final taxol concentration to 1, 5, and 20  $\mu$ M, respectively. At the end of the assembly reaction, the microtubule solution is chilled on ice.

The taxol-induced polymerization of tubulin is carried out in a standard microtubule assembly buffer (Pipes buffer, pH 6.8 [BRB80]). Because coupling of microtubules to the activated agarose matrix (see below) takes place inefficiently at pH 6.8, the pH of the microtubule solution is adjusted to 7.6 by addition of small aliquots of 2 M KOH just before coupling.

### Construction of Microtubule Affinity Columns

The agarose matrix used for construction of microtubule affinity columns consists of a 1:1 mixture of affigel 10 (Bio-Rad Laboratories, Cambridge, MA) and Sepharose CL6B (Pharmacia Fine Chemicals, Piscataway, NJ). Affigel 10 is an agarose matrix activated for coupling to protein by the presence of *N*-hydroxysuccinimide groups, and the CL6B is an inert agarose matrix that is included to create a more porous column with improved flow properties (Miller and Alberts, 1989). We generally construct columns in sterile plastic syringes (Becton Dickinson & Co., Mountain View, CA) fitted with polypropylene discs (Ace Glass, Inc., Vineland, NJ) as bed supports. The cross-sectional area of the column is increased according to the column volume using a 6-ml syringe for a 3-ml column, a 12-ml syringe for a 6-ml column, and so on.

An 18-gauge syringe needle fitted with a syringe of appropriate size is pushed through a rubber stopper on top of a filter flask to which suction can be applied. Equal settled volumes of CL6B and affigel 10 are poured into the syringe and washed several times with water at  $4^{\circ}\text{C}$ , with periodic stirring. Care is taken not to draw air into the column matrix throughout these and the following procedures. After storage for 1 h at  $4^{\circ}\text{C}$  to inactivate the affigel resin partially (overcoupling of the microtubules leads to a significant decrease in the binding capacity of the affinity matrix), the column bed is washed twice with C buffer in the manner described above. After the third wash, the buffer is drawn down to a level just above the surface of the column bed and the column is removed from the rubber stopper and sealed at the bottom. About 0.5 column volumes of a 2–3 mg/ml solution of taxol-stabilized microtubules at pH 7.6 (see above) is added, followed by thorough stirring with a teflon rod. The column is left undisturbed for 4–15 h at  $4^{\circ}\text{C}$  to allow coupling to occur, and then washed with several column vol of C buffer containing 10 mM ethanolamine (added from a 3-M stock, redistilled, and adjusted to pH 8) to block unreacted groups, followed by C buffer containing 1.0 mM DTT and 0.5 M KCl (flow rate of 1–2 column vol/h). The washes are saved and assayed for protein to determine the amount of tubulin bound to the column.

Our procedure generally causes  $\sim 60$ – $75\%$  of the input microtubule protein to bind to the column. The columns are stored at  $4^{\circ}\text{C}$  in BRB80 containing 10% glycerol, 1 mM DTT, 5  $\mu$ M taxol, and 0.02% sodium azide, and can be used for at least five experiments over a 1-mo period without a detectable change in their properties. For controls, BSA columns were prepared as described by Miller and Alberts (1989). We did not construct tubulin dimer control columns because of difficulties in obtaining purified stable tubulin dimers (Wilson, 1970; Weingarten et al., 1974).

### Preparation of *Drosophila* Embryo Extracts for Microtubule Affinity Chromatography

The extracts for our affinity chromatography experiments are prepared from living 2–3-h collections of *Drosophila* embryos (i.e., embryos are between

0 and 3 h postfertilization). The embryos are collected, dechorionated, and washed extensively with distilled water as previously described (Miller and Alberts, 1989). They are then suspended in 10 vol of C buffer containing 0.05% NP-40 and protease inhibitor stock (1:100). PMSF is added to 1 mM and the embryos are homogenized by several passes of a motor driven teflon dounce homogenizer. (Selection of a loose fitting pestle prevents disruption of yolk granules.) The embryo homogenate is centrifuged for 10 min at 12,000 g, followed by 60 min at 100,000 g. A thin floating layer on the surface of the final supernatant is removed by aspiration, and DTT is added to 0.5 mM before loading the extract onto affinity columns (see below). All steps are carried out at 4°C.

### Microtubule Affinity Chromatography

Embryo extracts are loaded onto affinity columns at 0.5–1 column vol/h. After loading, the columns are washed with 3–4 column vol of CX buffer, and then eluted in succession with this buffer plus either 1 mM MgATP, 0.1 M KCl, or 0.5 M KCl. The wash and elutions steps are carried out at 2 column vol/h, and all chromatography steps are at 4°C.

After the protein concentration in each fraction is determined (Bradford, 1976), the peak fractions are pooled. The protein in each pool is precipitated with 10% TCA as described by Miller and Alberts (1989), resuspended in gel sample buffer (0.5 ml for the eluate from a 15-ml column), and neutralized with the vapor from a Q-tip soaked in ammonium hydroxide. The pellets are solubilized by incubation at 50°C for 30 min, followed by 100°C for 3 min; each is then analyzed by SDS-PAGE (Laemmli, 1970), using Coomassie blue staining to visualize protein bands.

### Cosedimentation of MAPs with Taxol-stabilized Microtubules

As an alternative to microtubule affinity chromatography, MAPs were isolated by virtue of their ability to cosediment with taxol-stabilized microtubules, using the procedures described by Vallee and Collins (1986). Briefly, 0–3-h embryos (dechorionated and washed as described above) are added to 2 vol of BRB80 buffer containing protease inhibitor stock (1:100). After PMSF is added to 1 mM, the embryos are homogenized at 4°C, and the extract is clarified as described earlier. The supernatant is supplemented with 0.5 mM DTT, 1 mM GTP, 20  $\mu$ M taxol, warmed to 25°C for 5 min, and then transferred to ice for 15 min to allow polymerization of the endogenous tubulin. The taxol-stabilized microtubules and their associated proteins are collected by centrifugation through a sucrose cushion (BRB80X containing 10% sucrose) at 48,000 g for 30 min at 4°C. The pellet is washed twice at 4°C by resuspension in 0.2 times the original extract volume of BRB80X buffer, followed by a second centrifugation through a sucrose cushion. MAPs are dissociated from the microtubules by resuspending the final pellet in BRB80X buffer containing 1 mM ATP and 0.5 M KCl (0.07 times the original extract volume) at 25°C. The microtubules are removed by centrifugation at 45,000 g for 15 min at 20°C, and the MAP-containing supernatant is saved. Release of the MAPs at 25°C prevents dissociation of tubulin subunits (see Vallee and Collins, 1986).

Isolation of MAPs in a pH 7.6 Hepes buffer (see text) is carried out in the same manner, except that the BRB80 buffer is replaced by C buffer, BRB80X buffer is replaced by CX buffer, and the release of the MAPs from microtubules is carried out at 4°C, since taxol-stabilized microtubules are more stable when exposed to ATP and KCl in the pH 7.6 Hepes buffer.

### Generation of Mouse Polyclonal Antibodies to Gel-Purified Proteins

We have produced mouse polyclonal antibodies using procedures modified from those of Amero et al. (1987). A total of  $\sim$ 1.6 mg of affinity-purified MAPs are separated on two preparative 7–12%, 14.5 cm  $\times$  22 cm, SDS-containing polyacrylamide gradient gels (0.82-mm thick). The gels are stained with Coomassie blue, destained for 0.5 h, and treated with a solution of 2% glutaraldehyde in water for 1 h at 25°C. The gels are then transferred to 7% acetic acid to complete the destaining procedure. After soaking in water to remove the acetic acid, bands of interest are excised with a razor blade and homogenized with a motor-driven teflon dounce homogenizer in the presence of a small amount of water. The homogenized gel bands are then lyophilized and resuspended in 1.5 ml of sterile PBS.

Each gel slice is used for a total of four immunizations given intraperitoneally with a 22-gauge needle and spaced at 2-wk intervals. The first immunization is with 0.45 ml of homogenized gel slice slurry and 0.1 ml

of complete Freund's adjuvant, while the remaining three immunizations use 0.34 ml of the slurry and 0.1 ml of incomplete Freund's adjuvant. (The adjuvant mixtures are warmed to 37°C and vortexed vigorously before loading into the syringe). Beginning 1 wk after the final immunization, the mice are bled intraorbitally every 7–10 d, and the sera are tested for the presence of specific antibodies. Mice are anesthetized with ether before all immunizations and bleeds.

### Western Blotting

Proteins were detected by western blotting according to standard procedures (Towbin, 1979). Approximately 0.15 mg of crude extract protein or affinity-purified MAPs are electrophoresed on a preparative minigel (8  $\times$  6  $\times$  0.5 mm) and then transferred from the gel to a sheet of 0.45  $\mu$ m porosity nitrocellulose (Schleicher & Schuell Inc., Keene, NH) in the presence of 25% methanol, 0.15 M glycine, 0.02% SDS (transfer for 90 min at 300 mA in a Hoefer electroblotting apparatus). The nitrocellulose sheet is incubated for 45 min in TBS containing 5% BSA and 0.02% sodium azide and then placed in a miniblotted apparatus (Immunetics, Cambridge, MA). Each lane of this miniblotted is loaded with 55  $\mu$ l of antibody diluted into TBS containing 0.05% Tween 20 detergent (Bio-Rad), 5% BSA, and 0.02% sodium azide, and the miniblotted is then placed on a rocker platform for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse secondary antibody is used to visualize protein bands. To reduce nonspecific background staining, this secondary antibody was preadsorbed to methanol-fixed embryos by diluting the antibody into  $\sim$ 1 ml of TBS containing 0.5 ml (settled volume) of methanol-fixed embryos. After mixing on a rotating wheel (60 min, 25°C), the soluble antibody is diluted to its final working concentration and used immediately.

### Fixation and Immunofluorescent Staining of *Drosophila* Embryos

Methanol is the fixative that best preserves microtubule structures in the *Drosophila* embryo (Warn and Warn, 1986; Kellogg et al., 1988). For immunofluorescence staining, the methanol-fixed embryos are incubated in PBS containing 5% BSA, 0.05% Tween 20, and 0.02% sodium azide for 20 min. The embryos are then incubated overnight at 4°C in a 1:400 dilution of immune mouse serum in PBS containing 5% albumin, 0.1% Tween, and 0.02% sodium azide. These embryos are washed and treated with rhodamine-conjugated secondary antibody as described by Karr and Alberts (1986).

### Affinity Purification of Antibodies

Affinity purification of antibodies using nitrocellulose-bound antigen was carried out according to procedures described by Smith and Fisher (1984), with modifications. Affinity-purified MAPs are resolved on two preparative minigels and transferred to nitrocellulose sheets. The location of the desired protein band is determined by using a miniblotted to treat the lanes on both edges of the nitrocellulose sheet with primary antibody. After staining with alkaline phosphatase-conjugated secondary antibody, the central strip of unstained nitrocellulose that corresponds to the desired protein band is excised and cut up into small pieces, which are placed into a 1.5-ml tube. We used the 190-kD band to affinity purify the SI-24 antibody, the 105- and 89-kD bands for the SI-24 antibody, and the 190-kD band for the S5-47 antibody (see Fig. 6A). These pieces are washed with PBS and incubated for 30 min at 25°C in PBS containing 5% BSA, 0.1% Tween 20, and 0.02% sodium azide. The nitrocellulose pieces are then incubated in the primary antibody (0.5 ml of a 1:500 dilution of serum) for 1.5 h at 25°C with gentle mixing. After several 5-min washes in TBS containing 0.05% Tween 20, the antibody is eluted from the nitrocellulose with two 30-s washes of 0.25 ml of 0.1 M glycine, pH 2.3, 0.4 M NaCl, 100  $\mu$ g/ml BSA, 0.05% Tween 20, accompanied by gentle vortexing. The washes are pooled and immediately neutralized by addition of 0.5 ml PBS containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>.

The affinity purification procedure is repeated once more, using the same diluted primary antibody and nitrocellulose pieces. The eluted antibodies from both rounds of purification are pooled (2.0 ml total) and 0.05 ml of a 5% BSA solution is added as carrier. A centricon ultrafiltration device (Amicon Corp., Danvers, MA) is used to change the buffer to PBS and to reduce the volume of the antibody solution to 0.1 ml. The final 0.1-ml aliquot was used directly to stain  $\sim$ 100 embryos in a single well of a 96-well microtiter dish. The yield of antibody is dependent upon how strongly it reacts with a band on Western blots. We obtained enough of the S5-47 antibody

**Table 1. Purification of Tubulin from *Drosophila* Embryos\***

Fraction	Volume (ml)	Total protein (mg)	Tubulin (mg)	Percentage of yield	Relative purification
Cleared lysate	330	5,115	~100	(100)	(1.0)
Phosphocellulose	450	4,275	~100	~100	1.15
DEAE cellulose	47	183	103	~100	28
Cycled tubulin	15	56	56	56	50

\* The starting material was 160 g of frozen 0–4 h *Drosophila* embryos. The amount of tubulin in each fraction was determined according to Bradford (1976) (cycled tubulin) or by Western blotting, using purified tubulin as a standard. For the procedures used, see Materials and Methods.

to carry out about five immunofluorescent stainings, whereas the amount of the SI-4 antibody that we obtained allowed only one staining before it was exhausted. Controls that used the apparent background bands at 210–230 kD in Fig. 6 A (0.1 M KCl elution) for affinity purification failed to select any antibody that stained centrosomes from the SI-4 or SI-24 antisera.

## Results

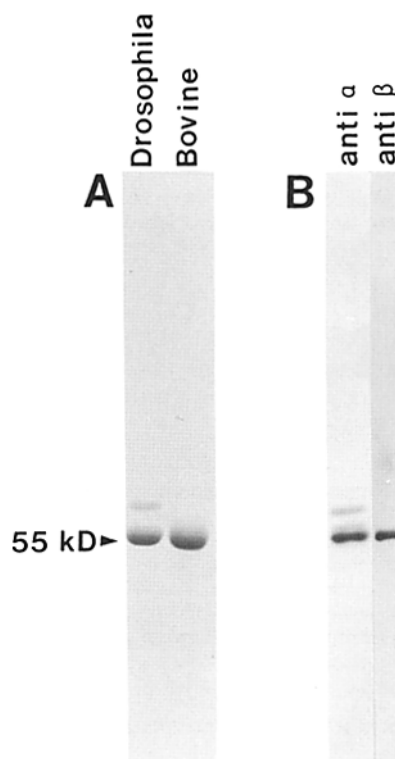
### Microtubule-Affinity Columns

We have constructed microtubule-affinity columns using methods modeled on those used to construct actin filament affinity columns (Miller and Alberts, 1989). The columns are constructed by covalently linking microtubules to an inert agarose support matrix, using tubulin purified from bovine brain (Mitchinson and Kirschner, 1984) or from early *Drosophila* embryos (Table I). Fig. 1 shows the result of polyacrylamide gel electrophoresis of the purified tubulins used for column construction. The more slowly migrating second band seen in the purified *Drosophila* tubulin is a variant form of the  $\alpha$ -tubulin subunit, as revealed by Western blotting (Fig. 1 B). This band may correspond to the highly divergent  $\alpha$ -4 tubulin identified by Theurkauf et al. (1986).

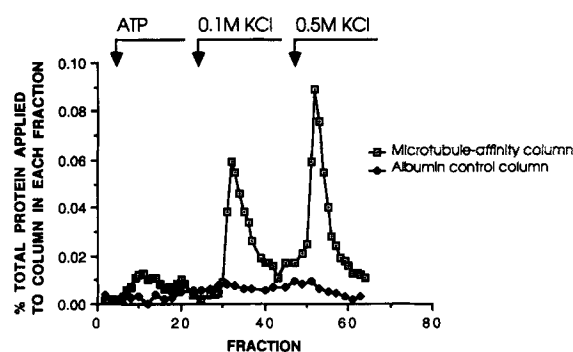
Microtubules are highly labile structures, and they must be stabilized to survive the conditions required for an affinity chromatography experiment (for example, low temperatures and high salt concentrations). Taxol, a low molecular mass plant toxin, binds tightly to microtubules and confers suitable stability (Horwitz et al., 1982). We routinely construct microtubule-affinity columns with bed volumes ranging from 2 to 20 ml that contain approximately 1 mg/ml of bound microtubules (see Materials and Methods). The columns can be reused at least five times over a 1-mo period without detectable changes in their properties. A control column is constructed by coupling BSA to the same agarose matrix.

### Affinity Purification of MAPs from the Early *Drosophila* Embryo

A cytoplasmic extract is made from early *Drosophila* embryos under conditions that solubilize >90% of the endogenous tubulin, making it likely that most of the proteins bound to microtubules (MAPs) are solubilized by the extraction procedure. The affinity columns are loaded with the clarified extract, washed extensively with buffer, and then eluted in three steps with CX buffer containing 1.0 mM MgATP, 0.1 M KCl, and 0.5 M KCl, respectively. Fig. 2 is an elution profile comparing the protein obtained from a bovine microtubule-affinity column and an albumin control column. Whereas 1.2% of the total extract protein binds to the microtubule



**Figure 1.** SDS polyacrylamide gel and immunoblot analysis of the tubulins used for construction of microtubule-affinity columns. Purified tubulins were resolved on SDS-containing 8.5% polyacrylamide gels (Laemmli, 1970) and visualized by Coomassie blue staining (A). Immunoblot analysis was carried out by transferring the resolved *Drosophila* tubulin bands to nitrocellulose and probing with antibodies against either alpha or beta tubulin (B). Alkaline phosphatase-conjugated secondary antibody was used to visualize antibody staining. We did not use a high pH running buffer to resolve the alpha and beta subunits of tubulin (Gard and Kirschner, 1985) because these conditions gave poor resolution of the slow-migrating *Drosophila*  $\alpha$ -tubulin band.



**Figure 2.** An elution profile comparing the amount of protein eluted from a microtubule-affinity column (bovine) and an albumin control column. In this experiment, 15-ml columns were used and 30 ml of extract was loaded onto each column. The columns were eluted with ATP, 0.1 M KCl, and 0.5 M KCl. The amount of protein in each fraction was determined (Bradford, 1976) by using BSA as a standard. The volume of each fraction is 1.5 ml; the protein concentration in the peak fractions from the salt elutions of the microtubule-affinity columns range from 40 to 80  $\mu$ g/ml.

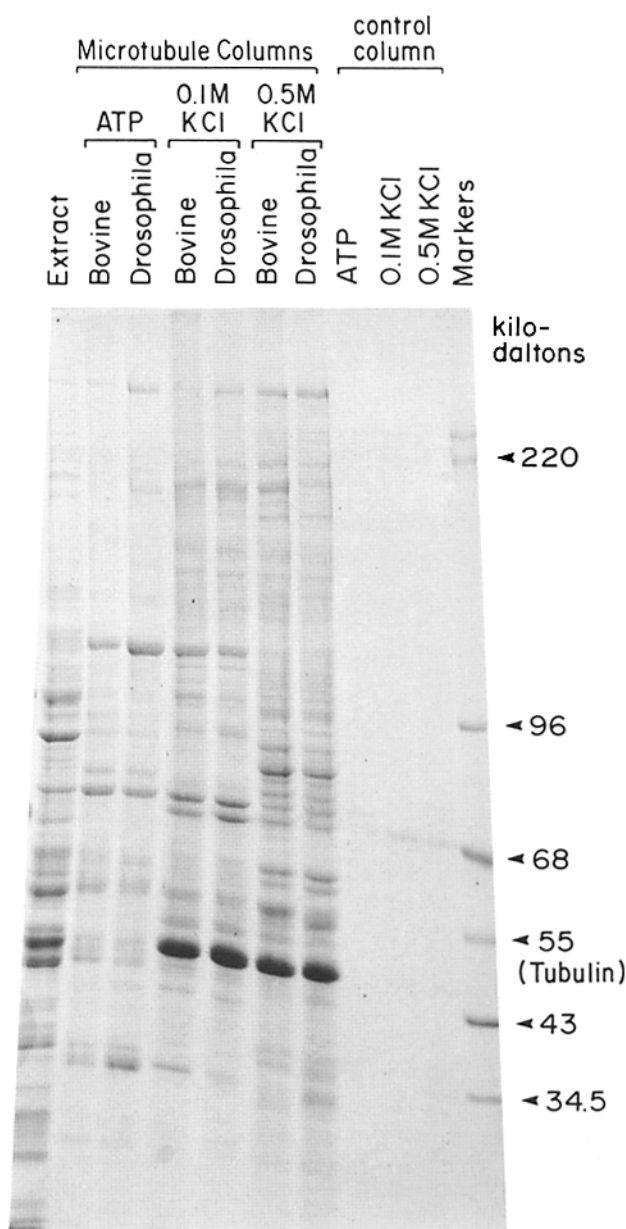
column, <0.1% binds to the control column, indicating that specific interactions with microtubules are observed.

To examine the proteins that elute from each of the columns, the column fractions were concentrated by TCA precipitation. The proteins were then separated by electrophoresis through an SDS-containing polyacrylamide gel and visualized by Coomassie blue staining (Fig. 3). A large number of different proteins bind specifically to *Drosophila* and bovine microtubule affinity columns, whereas very little binding is observed to an albumin control column. Moreover, the eluates are not contaminated with any of the major proteins in the extract applied to the columns. Greater than 90% of the total protein that elutes from the microtubule affinity columns, including all of the major species, will rebind to the columns after dialysis back into a low salt buffer, suggesting that the majority of these proteins bind directly to microtubules or are components of tightly associated multiprotein complexes that bind to microtubules (data not shown). Because the proteins eluting from the columns constructed with bovine and *Drosophila* microtubules are largely identical, it appears that most of the binding sites for MAPs on tubulin are conserved between these two species.

For comparison, we have also isolated MAPs from *Drosophila* embryo extracts by a procedure based on cosedimentation of MAPs with taxol-stabilized microtubules (Vallee and Collins, 1986). In this procedure, taxol is added to a concentrated cytoplasmic extract to polymerize the endogenous tubulin. Microtubules and their associated proteins are then collected in a pellet by centrifugation. After washing this pellet, MAPs are dissociated from the microtubules with a buffer containing 0.5 M KCl and 1.0 mM MgATP. Since the microtubule protein pellet forms clumps that are difficult to homogenize except in the presence of high salt, we were unable to elute MAPs from it with MgATP alone or with 0.1 M KCl to mimic more closely the microtubule affinity column elutions.

The proteins obtained when the cosedimentation procedure is used to isolate MAPs from a *Drosophila* embryo extract are analyzed by PAGE in Fig. 4. Comparison of these proteins (lane B) with the total proteins that are retained on a microtubule affinity column (lane D) reveals that a greater number of different MAPs are isolated by the affinity chromatography procedure. Moreover, the proteins isolated by the cosedimentation procedure constitute only 0.5% of the total extract protein, compared with 1.3% of the total protein isolated by affinity chromatography. It is possible that the yield of MAPs obtained by the cosedimentation procedure could be improved by the addition of more tubulin to the extract.

The published cosedimentation procedures for isolating MAPs use a pH 6.8 Pipes buffer. When affinity chromatography experiments are carried out in this buffer, significant amounts of tubulin are lost from the columns in the presence of high salt (data not shown). Our affinity chromatography experiments are therefore carried out at pH 7.6 in HEPES buffer (see Materials and Methods). When the cosedimentation experiment is repeated in the same buffers used for the chromatography experiments, the results more closely resemble those obtained by affinity chromatography. In both cases, the MAPs obtained constitute ~1.2% of the total extract protein, and the pattern of proteins obtained by each procedure is more similar (compare lanes C and D in Fig.

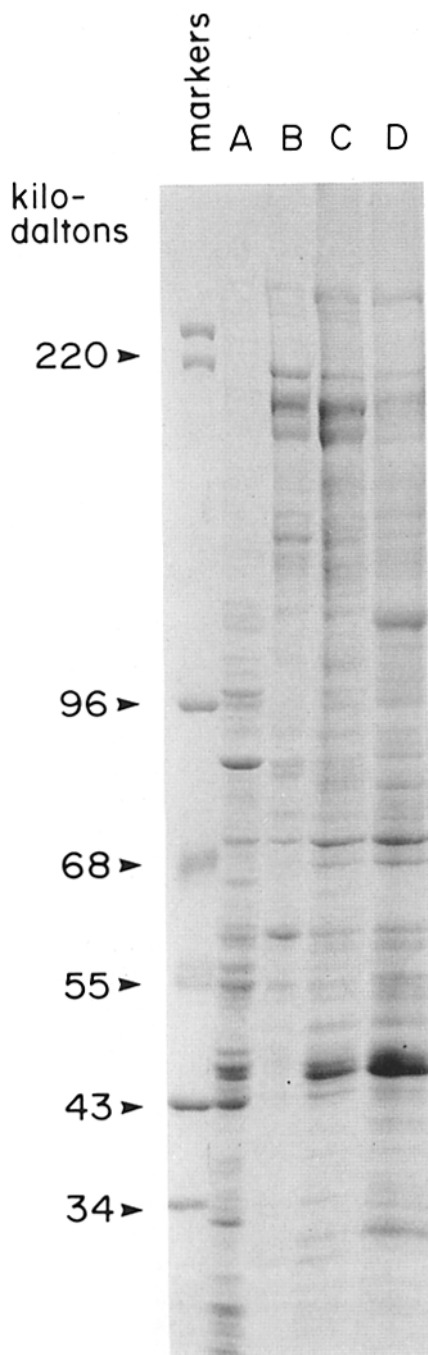


**Figure 3.** Analysis by SDS-PAGE of the proteins retained on *Drosophila* and bovine microtubule affinity columns and an albumin control column. The columns were loaded with a *Drosophila* embryo extract and eluted with ATP, 0.1 M KCl, and 0.5 M KCl, as in Fig. 2. The proteins in each fraction were resolved by electrophoresis through a 7–10% polyacrylamide gradient gel, and visualized by Coomassie blue staining. The mass of each marker protein is indicated on the right margin.

4). Although most of the major MAPs are enriched by both procedures, there are large differences in the relative amounts of various proteins, and many of the minor bands are different. Each procedure has advantages: although the cosedimentation procedure is more rapid and convenient, affinity chromatography allows better control of the elution conditions.

#### **Generation of a Library of Polyclonal Antibodies Against *Drosophila* MAPs**

To begin a characterization of the many proteins that bind to



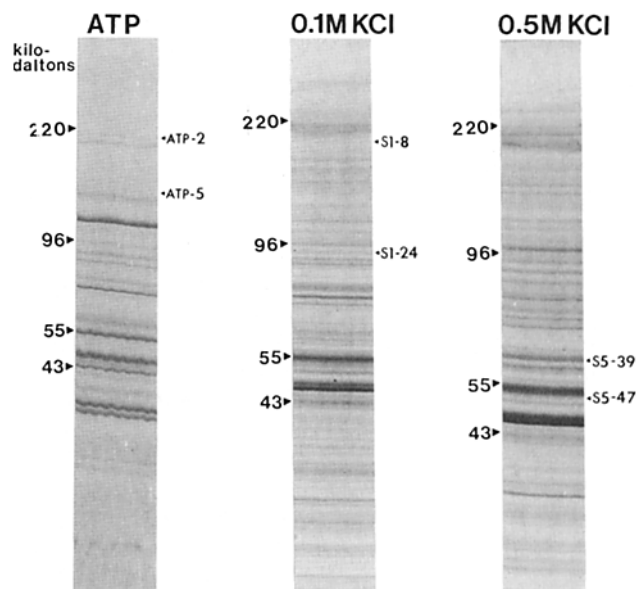
**Figure 4.** Comparison by SDS-PAGE of the MAPs isolated by microtubule affinity chromatography with the MAPs isolated by cosedimentation with taxol-stabilized microtubules. The proteins are resolved on a 7–10% polyacrylamide gradient gel and have been visualized by Coomassie blue staining. (Lane A) High speed supernatant. (Lane B) The MAPs isolated by cosedimentation in a pH 6.8 Pipes buffer. (Lane C) The MAPs isolated by cosedimentation in a pH 7.6 Hepes buffer. (Lane D) The total MAPs retained on a *Drosophila* microtubule affinity column.

microtubule affinity columns, we have generated a library of mouse polyclonal antibodies that recognize a large number of them. By means of such antibodies, the proteins obtained in our chromatography procedure can be localized in *Drosophila* embryos by immunofluorescent staining, so that

those associated with microtubules *in vivo* can be identified. Moreover, MAPs with particularly interesting localizations can be identified and selected for further study, using the antibodies as probes to aid in their further purification and characterization.

In several studies, it has been found that mice will generate an immune response against only the few most abundant and/or immunogenic of the proteins in a complex mixture, limiting the range of the monoclonal antibodies that can be prepared with impure antigens (Miller, K., D. R. Kellogg, and B. M. Alberts, unpublished results; Burke et al., 1982). We have therefore purified our MAPs to homogeneity by preparative PAGE, so as to be able to use each purified protein as an antigen. Mice will generate remarkably specific polyclonal antisera when immunized with a single protein (see below). Hence, the sera may be used directly for immunofluorescence and Western blotting. In addition, because mice can be immunized and bled with relative ease, large numbers of sera can be rapidly screened for the presence of antibodies that give particularly interesting immunofluorescence patterns.

Fig. 5 shows portions of the preparative gradient gels that we used to resolve the proteins that elute from microtubule affinity columns with ATP, with 0.1 M KCl, and with 0.5 M KCl. There are ~40 protein bands of molecular mass >25,000 that can be resolved in the ATP elution, and ~70 protein bands in each of the 0.1 M KCl and 0.5 M KCl elutions. Each of these proteins is assigned an identification code according to its elution behavior and relative molecular mass. For example, the protein designated ATP-1 is the protein of greatest apparent molecular mass that elutes from



**Figure 5.** Separation of affinity-purified MAPs by preparative PAGE. Only a portion of each gel slab is shown. Several of the protein bands that were excised and used for antibody production are identified on the right side of each gel, where a sample of the nomenclature used to identify each gel band is given. Note that protein bands are numbered starting at the top of each gel slab; thus, for example, ATP-2 is the band of second greatest apparent molecular mass in the ATP-eluting fraction, and S5-47 is a band of relatively low molecular mass eluting with 0.5 M KCl.



microtubule affinity columns with ATP, and the protein designated S5-1 is the largest protein that elutes with 0.5 M salt. Thus far we have immunized mice with eight of the ATP-eluting proteins, 24 of the 0.1 M KCl-eluting proteins, and 18 of the 0.5 M KCl-eluting proteins (see examples marked on Fig. 5). As a control, five mice were subjected to the same immunization protocol with polyacrylamide containing no protein.

Sera derived from these 50 immunized mice have been screened for the presence of specific antibodies by both Western blotting and immunofluorescence. Preimmune sera from 10 mice showed no response when tested by immunofluorescence. In addition, sera from the five mice subjected to a control immunization protocol showed no response when tested by Western blotting. Although several of these control sera produced a weak centrosomal staining when tested by immunofluorescence on whole fixed embryos, this staining persisted only for 2–3 wk after the final immunization. The experimental mice were therefore scored as reacting positively to an injected protein only if the serum from the mouse maintained a prolonged (>6-wk) titer of an antibody that produced a distinct immunofluorescence staining pattern and/or identified an affinity-purified protein by Western blotting. By these criteria, 24 of the 50 mice injected with a protein band generated an immune response against the injected protein (19 of which were positive by Western blotting). Several mice generated an immune response to proteins that were barely detectable by Coomassie blue staining. We estimate that in these cases we immunized the mice with a total of only 10–25 µg of protein.

Western blotting data for some of the antibodies are shown in Fig. 6. Serum from each mouse was diluted 1:400 and then tested against both microtubule affinity column fractions (Fig. 6 A) and against the crude extract that was loaded onto the affinity columns (Fig. 6 B). Some of the antibodies recognize more than one protein band; this could be because several proteins share the same epitope; more likely, however, it is attributable to proteolysis. We have therefore used arrow heads in Fig. 6 to indicate the band that is of the same molecular mass as the protein band originally injected into the mouse as antigen. In those few cases where only protein bands of different molecular mass than the injected band are detected, we assume that our initial immunization was carried out with a minor proteolytic fragment or that the antibody recognizes an epitope shared by more than one protein. In addition, some of the antibodies that work well for immunofluorescence react only weakly with a protein band on Western blots. This required us to allow the color development reaction to proceed for longer than usual, resulting in a substantial background. The background protein bands are those that are found across all of the lanes in each group (see legend to Fig. 6).

The weak signal observed for some antibodies is not because of a low antibody concentration, since the same signal is obtained over a wide range of dilutions, and the antibodies work well for immunofluorescence. We suspect that in some cases the signal is weak because of poor transfer of proteins to nitrocellulose. This may also explain why some of the antibodies that work well for immunofluorescence do not identify proteins by Western blotting. Antibodies that gave no reaction on Western blots are not shown in Fig. 6; 5 of the 24 antibodies in our library are in this category.

The Western blotting results in Fig. 6 demonstrate that the majority of the sera recognize proteins that are greatly enriched in the microtubule affinity column fractions, as compared to the starting extract (compare A with B). These blots also demonstrate the specificity of mouse polyclonal antibodies, since there is little or no background staining of bands in the extract. The specificity of these antibodies makes them suitable for immunofluorescence localization of their cognate proteins in fixed preparations of *Drosophila* embryos.

### *The Subcellular Distribution of MAPs in the Early Drosophila Embryo*

We have used immunofluorescent staining of whole *Drosophila* embryos to study the subcellular distribution of the proteins recognized by the 24 antibodies just described. As examples of the results obtained, photomicrographs showing the immunofluorescent localization of the MAPs recognized by three of these antibodies are presented in Fig. 7, 8, and 9. These micrographs display a small area of the surface of an embryo at nuclear cycle 10, the first cycle after the nuclei reach the embryo cortex. In addition to the antibody staining, the DNA has been stained with a second fluorochrome to reveal the location of the nuclei and their stage in the nuclear cycle. The distribution of microtubules in the early *Drosophila* embryo has been discussed in other studies (Karr and Alberts, 1986; Warn and Warn, 1986; Kellogg et al., 1988).

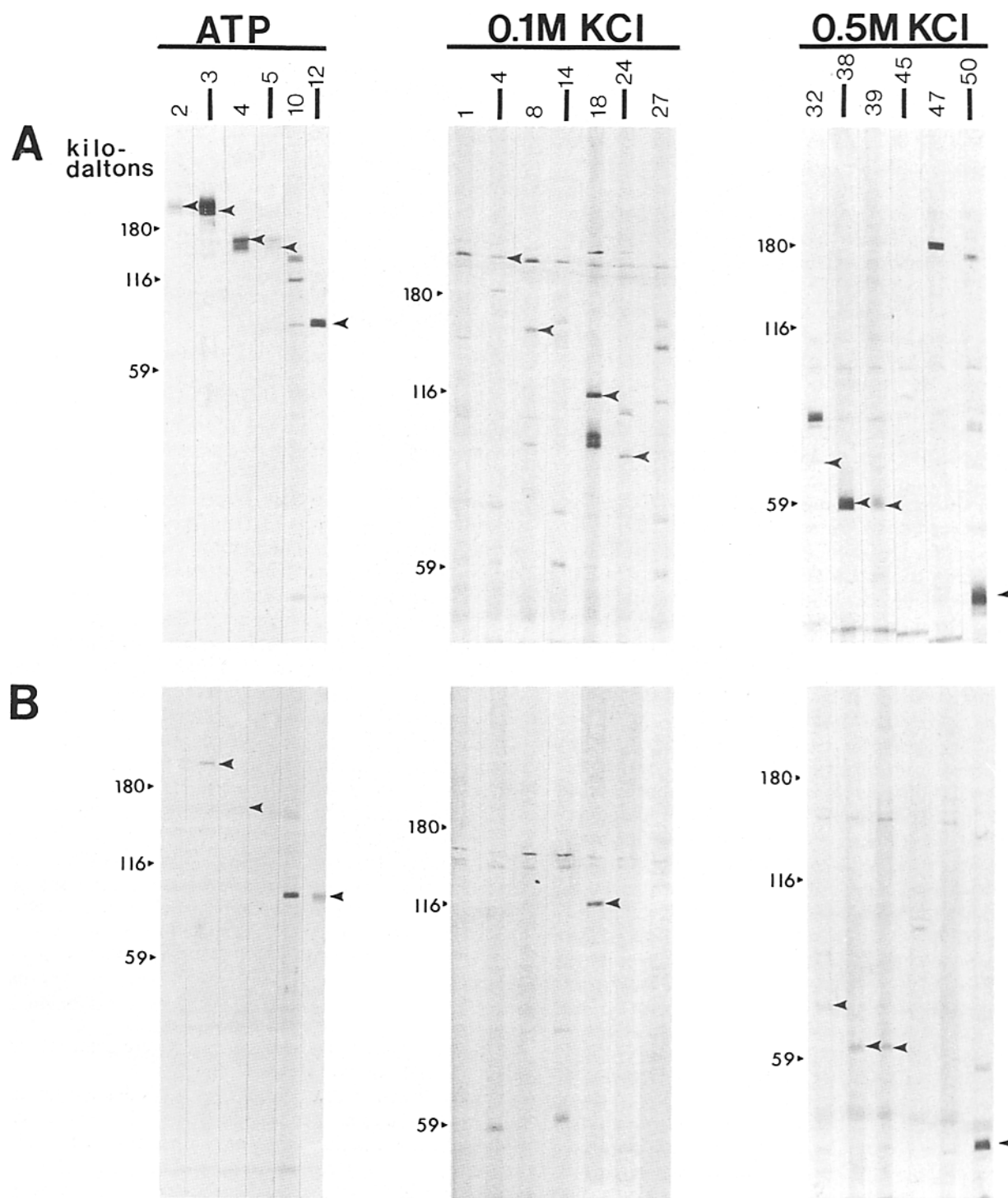
The S1-4 antibody most strongly recognizes a 190-kD protein that elutes from microtubules with 0.1 M KCl. It stains each centrosome as a bright dot throughout the nuclear cycle. Examples of the staining at metaphase (Fig. 7 A) and telophase (Fig. 7 B) are shown. The staining is brightest during anaphase and telophase, and is weakest during interphase.

The S5-39 antibody recognizes a 59-kD protein that elutes from microtubules with 0.5 M KCl. It stains a diffuse and irregular region around each centrosome at prophase (not shown). This staining persists through mitosis, but, in addition, a punctate staining appears at the metaphase plate that suggests kinetochore staining (Fig. 8 A). The staining at anaphase confirms the kinetochore localization. At this stage, one can see localization to the centrosomal region, as well as clear localization to the kinetochore region of the separating chromosomes (Fig. 8 C). There is little or no staining at interphase.

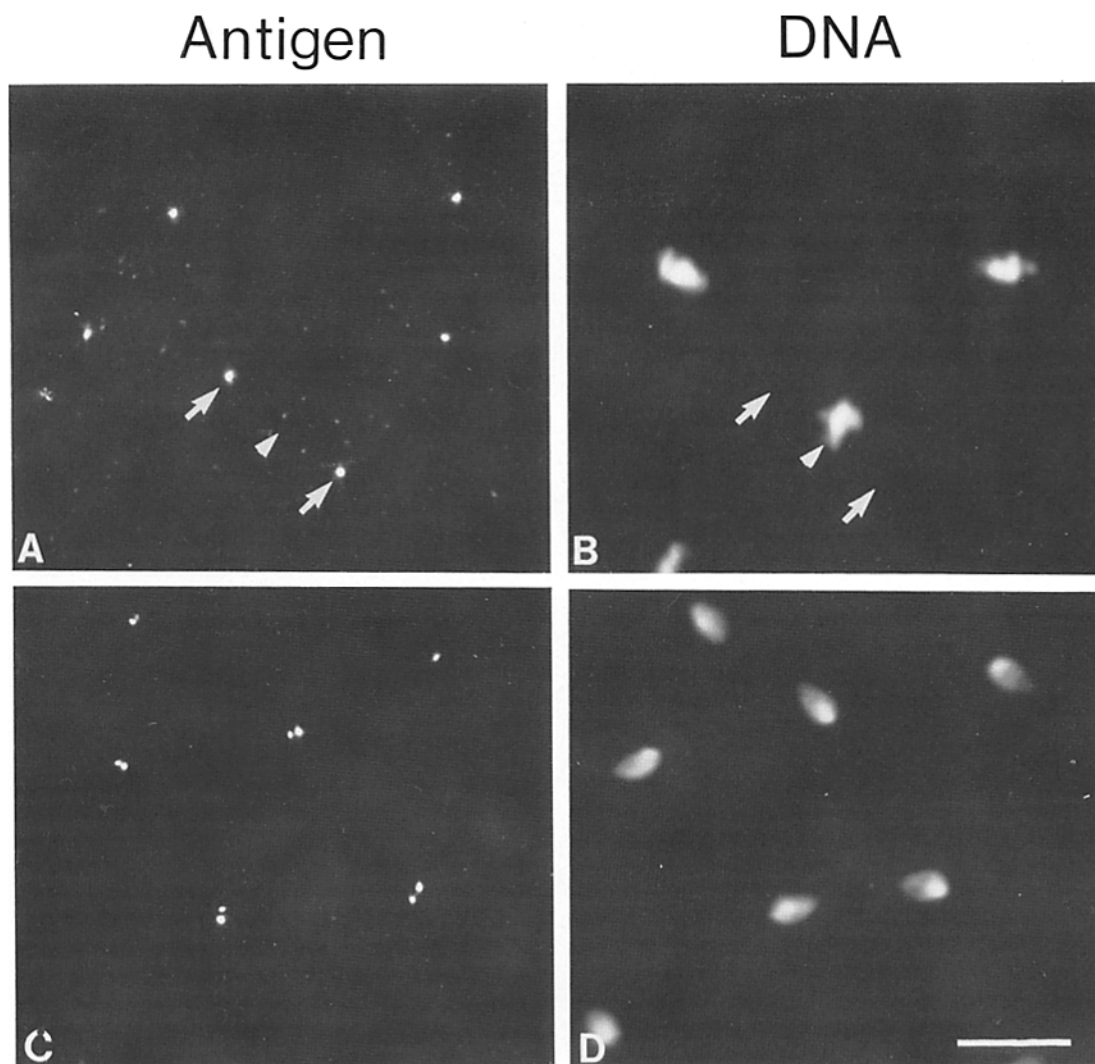
Finally, the distribution of the S1-8 protein (a 175-kD protein that elutes from microtubules with 0.1 M KCl) is illustrated at prometaphase, metaphase, and telophase in Fig. 9. At prometaphase, the antibody appears to stain a series of small dots that are either on the nuclear envelope or the outer edge of the nucleus (Fig. 9 A). The antibody also stains the centrosome at prometaphase, but this is not visible in the focal plane shown in Fig. 9 A. The antibody continues to stain the centrosome at metaphase, but in addition shows a diffuse localization around the spindle (Fig. 9 C). At telophase, the antibody stains both the centrosomal region (arrows, Fig. 9 E) and a region in the middle of the interzonal microtubules (arrowhead). During interphase there is little or no visible staining.

Similar data have been obtained for all of the antibodies in our library. The results are summarized in Table II, which lists the molecular mass and relative abundance of the pro-





**Figure 6.** Analysis by Western blotting of the antibodies raised against affinity purified MAPs. Procedures are discussed in Materials and Methods. Each antibody has been blotted against both the appropriate affinity column fractions (*A*) and against the crude extract (*B*). The blots that compare the affinity column fractions versus the crude extract were allowed to develop in the alkaline phosphatase substrate solution for equal amounts of time, so that the intensities of bands could be compared directly. We tested a total of 23 different mouse sera by Western blotting against the 0.1 m KCl-eluting proteins. All of these sera stained the group of bands in the 210–230 kD region (0.1 M KCl elution), even though 13 of the sera showed no reactivity by immunofluorescence and no specific reactivity by Western blotting. We conclude that these high molecular mass bands represent background staining, a conclusion supported by their failure to affinity purify any centrosome-staining antibodies from several antisera tested (see Materials and Methods). Since Fig. 6 represents a composite of several Western blots, the molecular masses indicated to the left of each set of lanes are approximate (within 10 kD); the exact molecular masses of the protein bands recognized by each of the antibodies are indicated in Table II. An arrowhead is used to indicate the band that is of the same molecular mass as the protein band originally injected into the mouse as antigen. Antibodies that gave no specific reaction on Western blots are not shown. See text for further discussion.

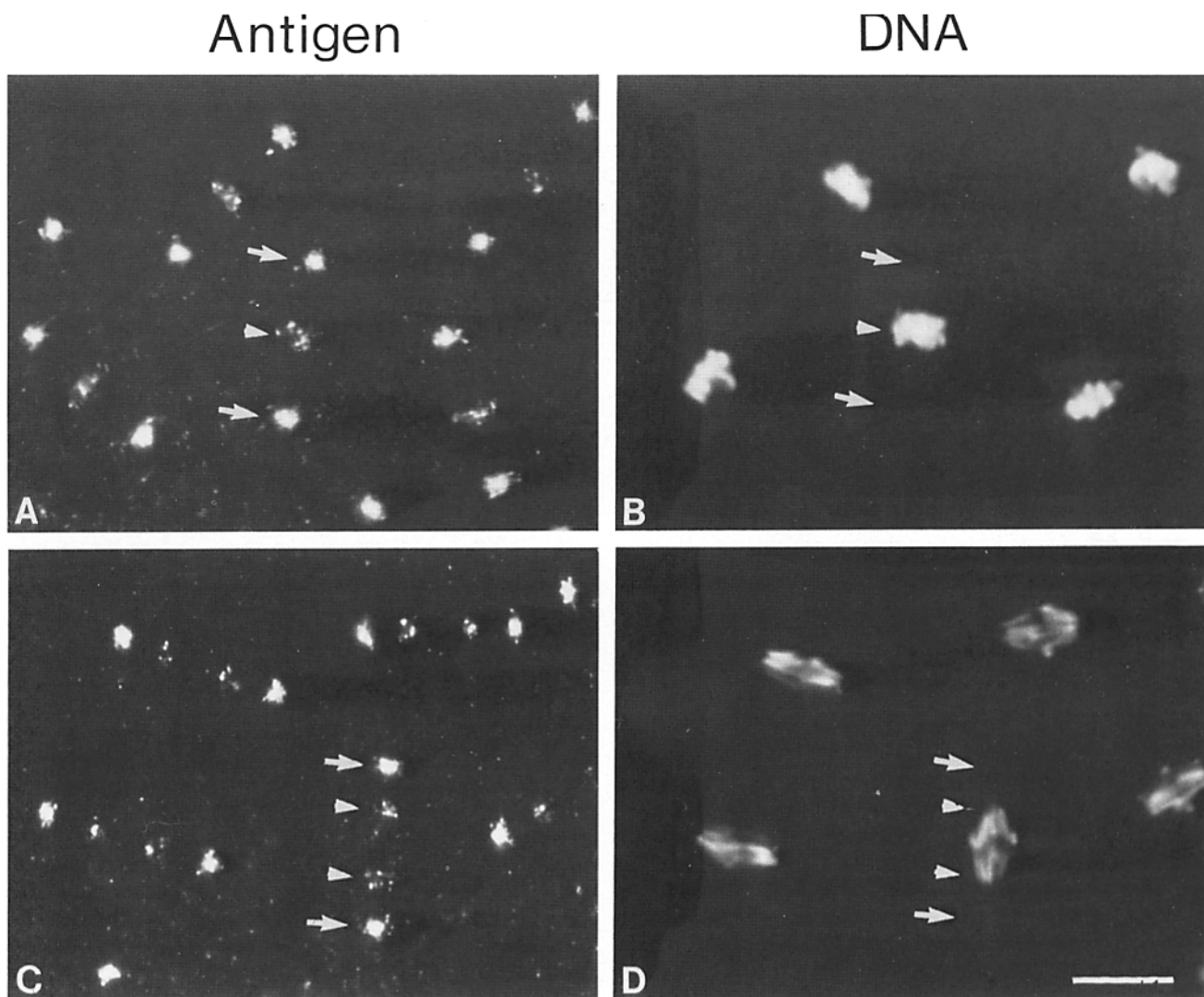


**Figure 7.** The distribution of the S1-4 antigen at metaphase (A) and telophase (C) of nuclear cycle 10, as revealed by immunofluorescence staining. The embryos have also been labeled with the fluorescent dye 4,6-diamidino-2-phenylindole to reveal the distribution of the DNA, and B and D show the distribution of the DNA in the region of each embryo corresponding to A and C, respectively. For clarity in A and B, the centrosomes corresponding to one mitotic spindle are indicated with arrows, and the position of the corresponding metaphase chromosomes is indicated by arrowhead. Bar, 10  $\mu$ m.

teins used as antigens, presents the results of Western blotting, and summarizes the immunofluorescence results. By immunofluorescence, 20 of the 24 antibodies that we have produced localize to microtubule structures in the early embryo. In addition, two of the remaining four antibodies appear to be localized exclusively to the neuronal system later in development, suggesting that they recognize MAPs confined to neuronal microtubules. In a similar study, Miller et al. (1989) immunized more than 40 mice with affinity-purified actin-binding proteins from the early *Drosophila* embryo. The sera from 26 of these mice were tested by immunofluorescence staining of embryos fixed according to the procedures used in this study, and none of the sera were found to stain microtubule structures. These observations demonstrate that the presence of serum antibodies that stain microtubule structures is dependent upon immunization with affinity purified MAPs, and they provide an additional control for the specificity of the antibodies described in this study. Our results suggest that nearly all of the proteins that

bind to our microtubule affinity columns are genuinely associated with microtubules in vivo.

Four of the antibodies recognize proteins that localize exclusively to the centrosome (S1-4, S1-20, S1-24, and S5-45), and seven of the antibodies recognize proteins that localize primarily to the centrosome (ATP-2, ATP-5, S1-1, S1-6, S1-25, S1-28, and S5-47) (see Fig. 7 and Table II). To prove that these are antibodies specific for MAPs enriched by microtubule affinity chromatography, we have used individual MAPs immobilized as SDS polyacrylamide gel bands transferred to nitrocellulose to affinity purify several of the centrosomal antibodies. The affinity-purified antibodies were then used for immunofluorescent staining of fixed embryos. For each of three antibodies tested (S1-4, S1-24, and S5-47), the affinity-purified antibody stained the centrosome in a pattern identical to the unfractionated sera. An example of the staining obtained with affinity-purified S5-47 antibody is shown in Fig. 10. These results confirm that the antibodies recognize centrosomal proteins that are enriched by microtubule affinity



**Figure 8.** The distribution of the S5-39 antigen at metaphase (A) and telophase (C) of nuclear cycle 10, as revealed by immunofluorescence staining. B and D show the distribution of the DNA in the corresponding region of the embryos in A and B. For clarity, the location of the centrosomes and chromosomes corresponding to one mitotic spindle are indicated with arrows and arrowheads, respectively. Bar, 10  $\mu$ m.

chromatography. Some of these proteins are likely to be functional components of the centrosome, whereas others may represent proteins that are translocated towards the minus ends of microtubules, thereby becoming localized to the centrosomal region.

There is a remarkable diversity in the subcellular distribution of the microtubule-associated proteins. Only a few of the 20 antibodies that stain early embryos recognize proteins with an identical intracellular location at all stages of the cell cycle, and only one has a distribution that closely mimics the distribution of tubulin. This is an unexpected result, inasmuch as the best characterized mammalian MAPs (relatively

abundant proteins initially isolated from brain) appear to be distributed along the length of most or all microtubules in cells (Vallee and Bloom, 1983; Bloom et al., 1984; Huber and Matus, 1984; Binder et al., 1985). The complexity of the microtubule cytoskeleton revealed here implies that we have a great deal yet to learn about these important filaments.

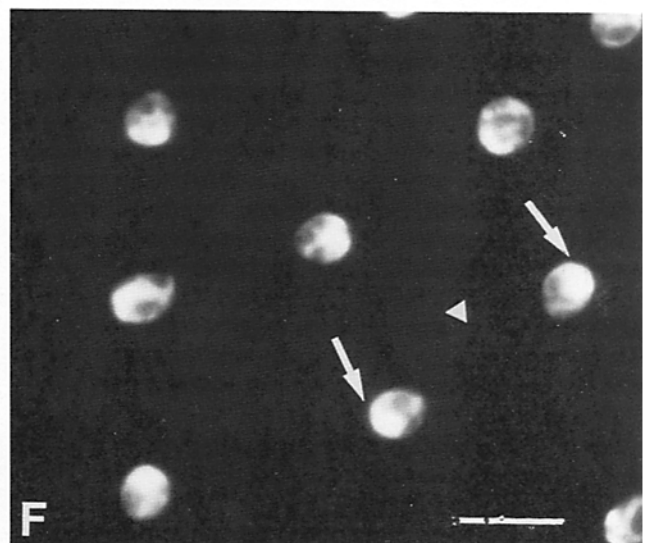
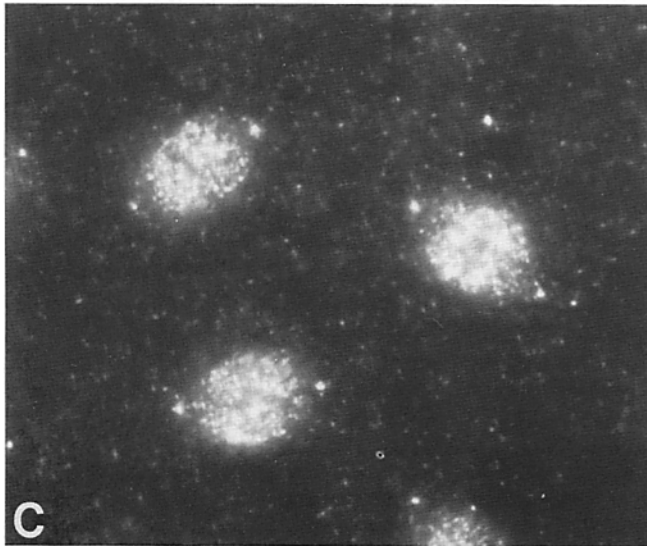
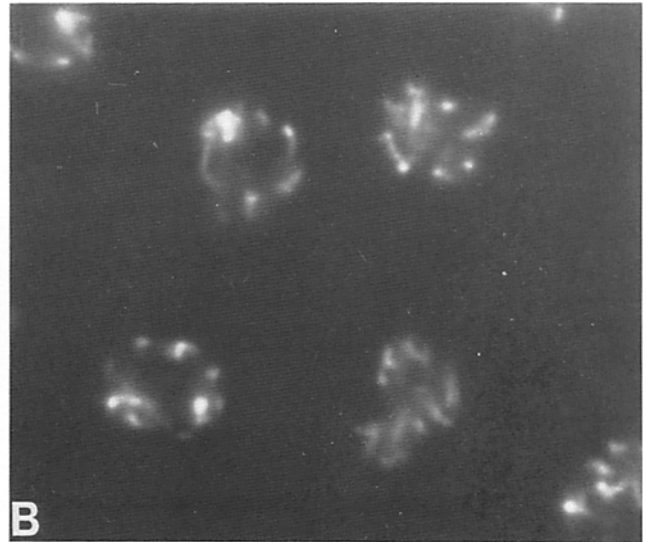
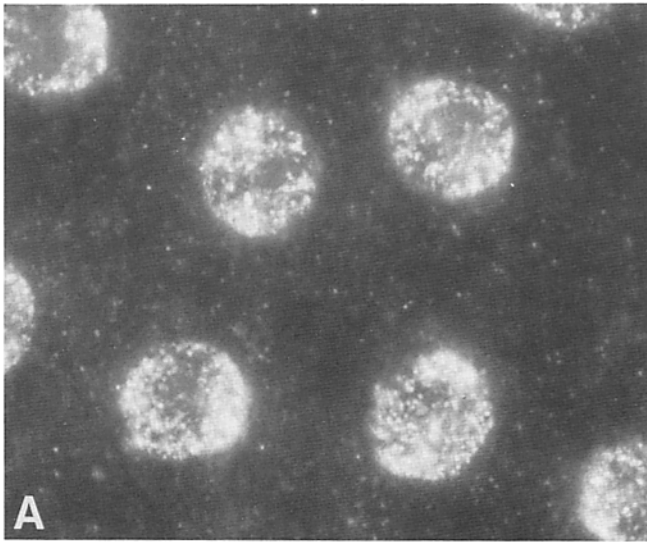
### Discussion

We have used microtubule affinity chromatography to isolate a large number of MAPs from the early *Drosophila* embryo. This method supplements the more conventional MAP puri-

**Figure 9.** The distribution of the S1-8 antigen at prophase (A), metaphase (C), and telophase (E), as revealed by immunofluorescence staining. B, D, and F show the distribution of the DNA in the corresponding region of the embryos in A, C, and E, respectively. At telophase, the antibody stains not only the centrosomal region (Fig. 9 E arrows), but also a region in the middle of the interzonal microtubules (arrowhead). There is no detectable staining in interphase. Bar, 10  $\mu$ m.

Antigen

DNA

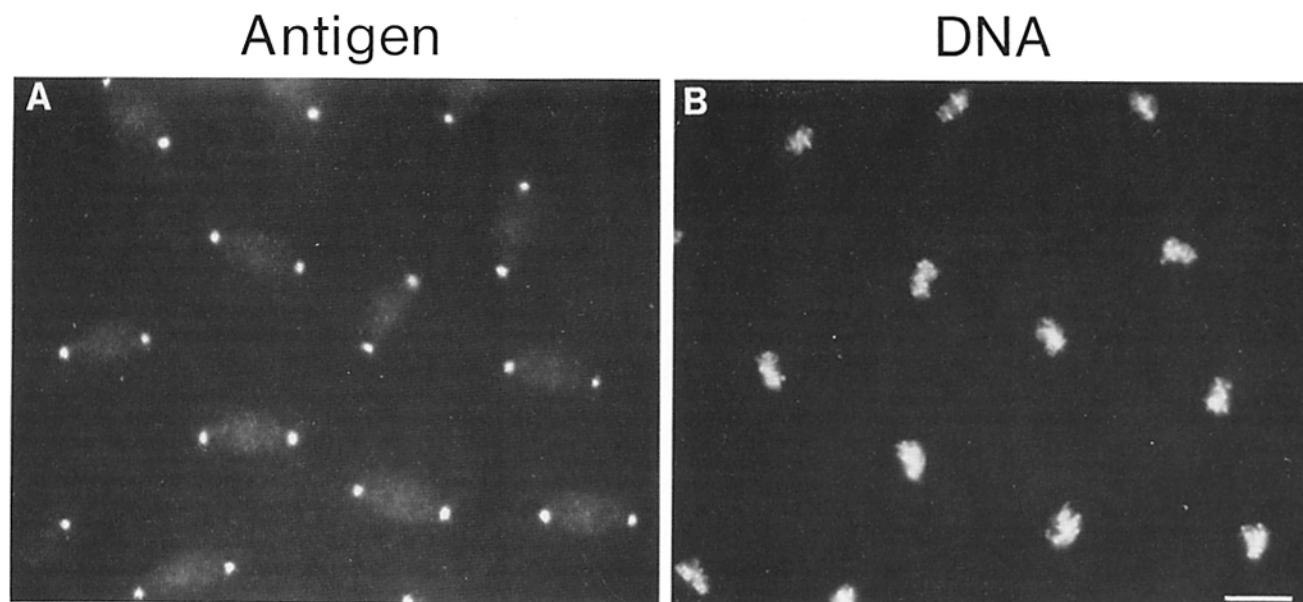


**Table II. Properties of the Antibodies Prepared by Immunization with Individual Protein Bands Isolated by Microtubule Affinity Chromatography**

Antibody	Molecular mass of antigen (kD)	Antigen abundance	Molecular mass on blots (kD)	Immunofluorescence distribution
ATP-2	205	++	205, 192	Strongly localized to the centrosomal region throughout the nuclear cycle. Weakly localized to microtubule arrays.
ATP-3	192	++	205, 192 (205)	Localized to most or all microtubules throughout the nuclear cycle.
ATP-4	184	+	164, 147 (164, 147)	Similar to ATP-3, except preferentially localized to astral microtubules at anaphase.
ATP-5	147	+	164, 147	Localizes to the centrosomal region throughout the nuclear cycle during preblastoderm divisions. Localizes to microtubule arrays during late cycle 14.
ATP-10	90	++	142, 107, 75 (75)	Cytoplasmic localization. Does not appear to be microtubule associated.
ATP-12	77	++	77 (77)	Diffuse blotchy localization near the spindle pole.
S1-1	335	+	157	Centrosomal localization from metaphase through telophase. Appears as a very fine dot. Very weak localization to the spindle.
S1-4	222	+	222,* 190 (60)	Centrosomal localization throughout the nuclear cycle. Appears as a fine bright dot, weakest during late interphase and early prophase. See Fig. 7.
S1-6	194	+++	—	Similar to S1-1.
S1-7	182	+	—	Localized to spindle during early metaphase, to spindle and centrosomes during late metaphase. Localized to centrosomes and interzonal microtubules during anaphase and telophase.
S1-8	175	+	175 103 93	Localized to the centrosome at all stages except interphase. Also localized to the nuclear envelope region at prophase, the spindle, and to a region in the middle of the interzonal microtubules. See Fig. 9.
S1-14	136	++	160 (60)	Similar to S1-7.
S1-18	110	+	110, 96, 92 (110)	Similar to S1-8, but localizes over all interzonal microtubules rather than to a central band during telophase.
S1-20	102	++	—	Centrosomal localization from metaphase through telophase. Appears as a cluster of fine dots.
S1-24	89	++	105 89	Centrosomal localization from anaphase through early interphase. Appears as a fine dot, somewhat weak.
S1-25	86	+++	—	Centrosomal localization from metaphase through telophase. Appears as a diffuse region, slightly punctate. Very weakly localized to the spindle.
S1-27	84	++	148, 109	Not detectable by immunofluorescence.
S1-28	79	++	—	Centrosomal localization throughout the nuclear cycle. Very weak localization to the spindle.
S5-32	68	++	82, 68 (82, 68)	Localized to centrosome at anaphase, telophase, and early cycle 14. Localized to DNA strongly during interphase and telophase, weakly during the remainder of the nuclear cycle.
S5-38	60	++	60 (60)	Neuronal?
S5-39	59	++	60 (60)	Localized to kinetochores during metaphase and anaphase. Centrosomal localization from prophase through telophase. See Fig. 8.
S5-45	52	+	93 (93)	Centrosomal localization throughout the nuclear cycle. Appears as a fine bright dot.
S5-47	50	+	190	Centrosomal localization throughout nuclear cycle. Very bright at anaphase/telophase, weak at interphase. Localized weakly to the spindle.
S5-50	46	+++	180, 80, 46 (180, 80, 46)	Neuronal?

Regular numbers indicate the molecular masses of protein bands recognized by Western blotting in the enriched microtubule affinity column fractions, whereas numbers in parentheses indicate the molecular masses of protein bands recognized in the crude extract. A minus sign indicates that no protein bands are detectable under the conditions that we have used for Western blotting.

\* The 222-kD band recognized by the S1-4 antibody is also recognized as a background band by other antibodies (see legend to Fig. 6). The S1-4 antibody most strongly recognizes the 190-kD band.



**Figure 10.** Immunofluorescent staining with affinity-purified S5-47 antibody. The antibody was affinity purified using a specific MAP gel band immobilized on nitrocellulose, as described in the text. Antibody staining is shown in Fig. 10 A, whereas Fig. 10 B shows the distribution of the DNA in the corresponding region of the embryo, as revealed by 4,6-diamidino-2-phenylindole staining. The embryo is in metaphase of nuclear cycle 10. Similar results have been obtained with antibodies S1-4 and S1-24. Bar, 10  $\mu$ m.

fication procedure in which proteins are bound to microtubules in solution, cosedimented with the microtubules, and then released from them with ATP and/or KCl (Vallee and Collins, 1986). Our column procedure has the advantage that low affinity interactions can be detected (Herrick and Alberts, 1972), and proteins that are present in small amounts can be greatly enriched by passage of a large amount of protein solution through the affinity column. Microtubule affinity columns can be reused multiple times, and they also provide a powerful purification step when a MAP has already been partially purified by conventional procedures. As expected, the proteins isolated by microtubule affinity chromatography and the proteins that cosediment with taxol-stabilized microtubules are similar, although there are some reproducible differences (Fig. 4). Accordingly, the optimal procedure to use should be determined empirically for each MAP. It is likely that microtubule affinity columns will be best for the isolation of MAPs having a weak affinity for microtubules, whereas MAPs that have a relatively strong affinity for microtubules will be more conveniently isolated by the cosedimentation procedure.

We have generated a library of mouse polyclonal antibodies that specifically recognize 24 of the affinity-purified MAPs, and we have used immunofluorescence staining of fixed *Drosophila* embryos to study the subcellular localization of the proteins recognized by each antibody. Because at least 20 of the 24 antibodies recognize proteins that are localized to microtubule-related structures in fixed embryos, the majority of the proteins that bind to microtubule affinity columns appear to be genuinely associated with microtubules in vivo. Extrapolation to the remaining proteins isolated on these columns allows us to estimate the number of different MAPs in the early *Drosophila* embryo. Approximately 180 different protein bands can be detected by Coomassie blue staining when affinity-purified MAPs are resolved on SDS-containing polyacrylamide gels. However,

there appears to be some overlap in the proteins found in different elutions, and some bands clearly represent proteolytic breakdown products or differently modified forms of the same protein (see Fig. 6). Even when these factors are accounted for, one is forced to conclude that there are at least 50–100 different MAPs in the early *Drosophila* embryo. This may even be an underestimate, since MAPs that are not solubilized by our extraction procedure would not be detected. Also, we have generally carried out our affinity chromatography experiments under conditions where microtubules become nearly saturated with bound MAPs. This should lead to a competition between proteins that bind to related sites on the microtubule lattice, which would favor the binding of proteins with a relatively high affinity for microtubules. Proteins that bind to microtubules with a lower affinity could be isolated by running the microtubule-affinity columns under subsaturating conditions, or by running extracts over two affinity columns, using the first column to remove the high affinity binding proteins. (Proteins with a  $K_d$  as high as  $10^{-5}$  M should be retarded by 1 column vol; see Herrick and Alberts, 1976.)

There is a striking diversity in the subcellular localization of these MAPs. Some are localized to the centrosome, while others localize to the spindle, kinetochores, interzonal microtubules, or combinations of these structures (Table II). These localizations can change dramatically through the nuclear cycle. The S1-8 antibody, for instance, stains the spindle at metaphase, while at telophase it stains only the centrosomes and a narrow region in the middle of the interzonal microtubules. The S5-47 antibody stains the centrosome very brightly during anaphase and telophase, but its antigen is barely detectable during interphase. Understanding the functional significance of these diverse localizations and how they are generated is likely to be important for understanding the structure and function of microtubule networks.

The proteins that localize to the centrosome are particu-



larly interesting to us, since they represent the first biochemically defined MAPs that localize uniquely to this important microtubule organizing center (for reviews, see Karsenti and Maro, 1986; Vorobjev and Nadezhdina, 1987). Previously, the centrosome has been characterized only by functional or morphological criteria (Mitchison and Kirschner, 1984; Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982), or by its staining with monoclonal and autoimmune antibodies (Calarco-Gillam et al., 1983; Moroi et al., 1983; Gosti-Testu et al., 1986; Whitfield et al., 1988). Combining microtubule-affinity chromatography with polyclonal antibody production would seem to open a new approach to studying the structure and function of the centrosome. Our antibodies that recognize centrosomal proteins should allow us to identify the corresponding genes from cDNA clone libraries in expression vectors, revealing the sequence of the protein and allowing conventional genetic approaches to centrosomal function in *Drosophila*. Moreover, the proteins produced by the cloned genes can be used to generate large amounts of polyclonal sera, which can be injected into early *Drosophila* embryos in an attempt to disrupt the function of the corresponding proteins in vivo. This kind of approach has been used successfully in other systems (Mabuchi and Okunono, 1977; Warn et al., 1987). It is especially promising in the case of the early *Drosophila* embryo, where the behavior of the highly dynamic microtubule networks can be observed in real time after injection of fluorescently labeled tubulin into the living cell (Kellogg et al., 1988). In addition, since large quantities of *Drosophila* embryos are readily available, the identified centrosomal components can be purified in sufficient quantities for their structures and activities to be characterized in vitro. Finally, a special opportunity to study centrosome multiplication and behavior independent of nuclear division is provided by injection of the DNA synthesis inhibitor aphidicolin into these embryos (Raff and Glover, 1988).

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